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Characterization of a Holliday Junction-Resolving Enzyme from *Schizosaccharomyces pombe*

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The rearrangement and repair of DNA by homologous recombination involves the creation of Holliday junctions, which are cleaved by a class of junction-specific endonucleases to generate recombinant duplex DNA products. Only two cellular junction-resolving enzymes have been identified to date: RuvC in eubacteria and CCE1 from *Saccharomyces cerevisiae* mitochondria. We have identified a protein from *Schizosaccharomyces pombe* which has 28% sequence identity to CCE1. The YDC2 protein has been cloned and overexpressed in *Escherichia coli*, and the purified recombinant protein has been shown to be a Holliday junction-resolving enzyme. YDC2 has a high degree of specificity for the structure of the four-way junction, to which it binds as a dimer. The enzyme exhibits a sequence specificity for junction cleavage that differs from both CCE1 and RuvC, and it cleaves fixed junctions at the point of strand exchange. The conservation of the mechanism of Holliday junction cleavage between two organisms as diverse as *S. cerevisiae* and *S. pombe* suggests that there may be a common pathway for mitochondrial homologous recombination in fungi, plants, protists, and possibly higher eukaryotes.

Holliday junctions, or four-way DNA junctions, are created when DNA is rearranged or repaired by homologous recombination. Strand exchange between two homologous DNA duplexes produces a Holliday junction, which can translocate by branch migration to yield recombinant heteroduplex DNA stretches. Duplex DNA products are resolved by a class of endonucleases specific for the four-way junction structure (reviewed in reference 41). Junction-specific resolving enzyme activities have been identified from a wide variety of sources including eubacteria, bacteriophage, eucarya and eucaryal viruses. Of the five junction-resolving enzymes cloned to date, three are of bacteriophage origin: T4 endonuclease VII (22), T7 endonuclease I (5), and lambdoid phage RusA (19). The major junction-resolving enzyme in the eubacteria is encoded by the *ruvC* gene, first identified in *Escherichia coli* (31). The sole eucaryal representative of the junction endonucleases which has been identified is the mitochondrial CCE1 protein from *Saccharomyces cerevisiae* (15). Comparison of the amino acid sequences of these five proteins reveals very little significant conservation of sequence, suggesting that the problem of four-way junction resolution has been solved by nature several times independently, perhaps by recruitment of nucleases with different specificities. Indeed the solution of the X-ray structure of RuvC identified a strong conservation with the fold of RNase H1 which was not apparent from inspection of the primary sequence (1). As the structures of other members of this family are solved, further evolutionary relationships may well come to light.

The best-characterized eukaryotic resolving enzyme is the *S. cerevisiae* CCE1 protein. The nuclear *CCE1* gene encodes a 41-kDa polypeptide which is targeted to the mitochondrion (10). *CCE1* is allelic to the *MGT1* gene, which was identified as a suppressor of hypersuppressive transmission of *rho* mitochondrial DNA (44). *cce1* yeasts have a large increase in the numbers of branched mitochondrial DNA (mtDNA) molecules, suggesting a role for the protein in the resolution of Holliday junctions linking mtDNA recombination intermediates (17). The protein was partially purified from yeast and shown to cut cruciform DNA and synthetic four-way junctions (9, 35). It was subsequently overexpressed in *E. coli* and purified to homogeneity (16, 42). CCE1 is a dimeric protein that binds four-way DNA junctions with a high degree of structural specificity. The enzyme exhibits a strong sequence preference for cleavage of junctions, cleaving most efficiently at the point of strand exchange after a 5′-CT sequence (42). CCE1 manipulates the stacked-X form of the four-way junction on binding, unfolding it into the square, open configuration that is found for free junction in the absence of added metal ions (43). Structural distortion of DNA junctions is induced by the binding of T7 endonuclease I (6), T4 endonuclease VII (25), and RuvC (2) and appears to be quite general for the junction-resolving enzymes.

With the exception of CCE1, very little is known about the enzymology of homologous recombination in eukaryotes. Although junction-resolving activities have been detected in *S. cerevisiae* (40), mammalian cells (8, 14), and vaccinia virus (34), these enzymes have not been purified to homogeneity and no sequence information is available. Extensive genetic screens for mutations affecting homologous recombination, primarily in *S. cerevisiae*, have also failed to identify convincing candidates for this role. This paper reports the identification, heterologous expression, and characterization of a second eucaryal four-way junction-resolving enzyme. The YDC2 protein of *Schizosaccharomyces pombe* exhibits weak sequence similarity to CCE1 and is shown to be a Holliday junction endonuclease. YDC2 has properties in common with both of the other cellular resolving enzymes studied so far: CCE1 and RuvC. It is likely that YDC2 fulfills the same role as CCE1 in the resolution of four-way junctions in recombining mtDNA. *S. pombe* and *S. cerevisiae* are highly divergent organisms; some estimates set the time of divergence as much as 1 billion years ago (33). Conservation of the mechanism of Holliday junction resolution between these two organisms suggests that there may be a common mechanism for mitochondrial homologous recombination in fungi, plants, and possibly higher eukaryotes.

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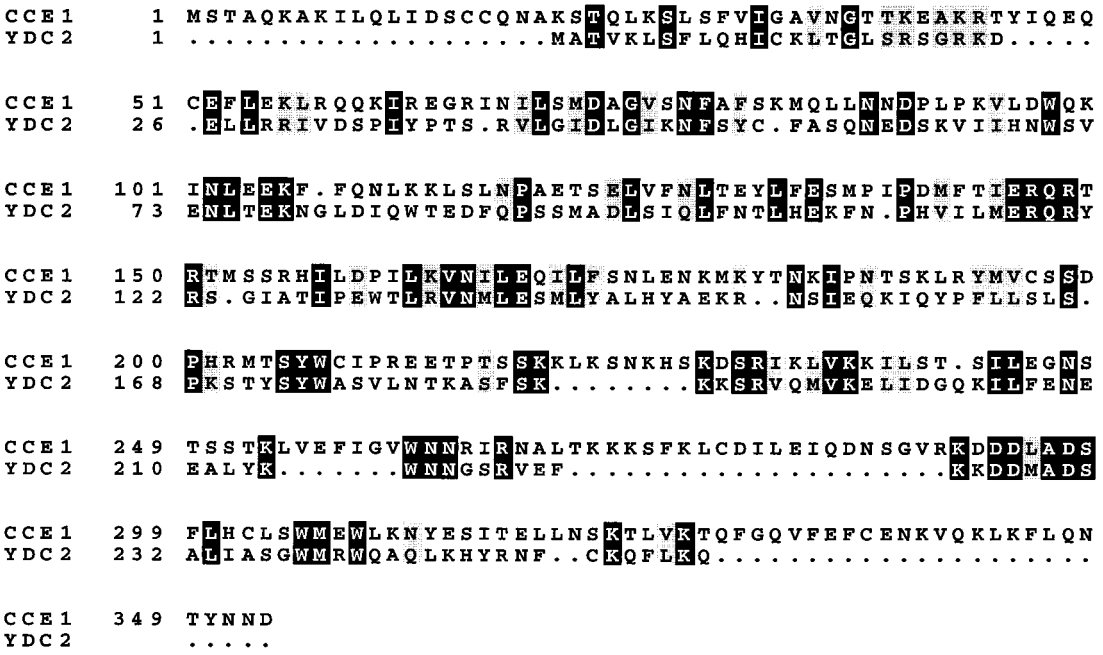

FIG. 1. Alignment of the CCE1 and YDC2 protein sequences. Sequences were aligned automatically with the program PILEUP (University of Wisconsin Genetics Computer Group) and shaded with the program BOXSHADE. Black boxes indicate sequence identities, and gray boxes indicate sequence similarities. The overall sequence identity between the two proteins is 28%.

MATERIALS AND METHODS

Cloning the *YDC2* gene. Standard protocols for the manipulation of DNA were followed (26). The *YDC2* gene was amplified from *S. pombe* 972 h^- chromosomal DNA by PCR. The oligonucleotide primers used for PCR were 5′-CGTCGGATCCCATATGGCCATGGCTACTGTGAAACTTAGTTTTTT ACAGC (5′ oligonucleotide) and 5′-GGCAGGATCCGTCGACCTATTGTT TCAGAAACTGTTTGCAAAAGTT (3′ oligonucleotide). The oligonucleotides introduced several restriction sites at either end of the amplified gene to facilitate subcloning. Amplified *YDC2* was subcloned into the *Bam*HI site of the pUC119 vector (Clonetech), creating plasmid pUC119-*YDC2*. Single-stranded pUC119-*YDC2* DNA for sequencing was produced with the helper phage MK407 (Pharmacia). The *YDC2* gene was subcloned from pUC119-*YDC2* into the *Bam*HI and *Sal*I sites of the pMALc2 expression vector (New England Biolabs), allowing expression of YDC2 as a fusion with maltose-binding protein (MBP).

Expression and purification of MBP-YDC2. YDC2 was expressed as a fusion protein with *E. coli* MBP from plasmid pMALc2 in *E. coli* DH5αF′I^q (Bethesda Research Laboratories). Cells were grown in Luria-Bertani medium containing 100 μg of carbenicillin per ml and 35 μg of kanamycin per ml in shaker flasks at 28°C to an absorbance at 600 nm of 0.5 to 0.8. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM, and the cells were incubated for an additional 2.5 h under the same growth conditions. The cells were harvested by centrifugation, subjected to one freeze-thaw cycle to aid lysis, and resuspended in 5 ml of lysis buffer (50 mM Tris · HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA, 0.2 mM dithiothreitol [DTT]) per g (wet weight) of cells. The cells were lysed by sonication (three 45-s bursts on ice), and the lysate was cleared by centrifugation (4°C for 20 min at 40,000 × *g*). The cleared lysate was subjected to ammonium sulfate fractionation. The protein precipitating between 25 and 65% saturation was resuspended in MBP buffer (20 mM Tris · HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA, 0.2 mM DTT), and applied to an amylose column (New England Biolabs) preequilibrated with MBP buffer. The column was washed with 2 volumes of MBP buffer to remove unbound material, and the fusion protein was eluted with 5 volumes of MBP buffer plus 10 mM maltose. The fusion protein was next applied to a POROS HS 20/100 sulfopropyl cation-exchange column (Perseptive Biosystems) preequilibrated with buffer A (10 mM morpholineethanesulfonic acid [MES; pH 6.0], 50 mM NaCl, 1 mM EDTA, 0.2 mM DTT) and eluted with a gradient of 0.05 to 1.0 M NaCl in buffer A. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (see Fig. 2) showed the presence of a band with the expected molecular mass of the MBP-YDC2 fusion: 70 kDa.

Cleavage of fusion protein and purification of YDC2. Purified fusion protein was cleaved with factor Xa (Promega) in buffer A at room temperature overnight, and the products were analyzed by SDS-polyacrylamide gel electrophoresis. Once an acceptable level of cleavage was apparent, the liberated protein was separated from MBP and any remaining fusion protein by chromatography. The cleavage products were applied to a POROS HS 20/100 column equilibrated with buffer A and eluted with a gradient of 0.05 to 1.0 M NaCl in buffer A. Under these conditions, MBP did not bind to the column and YDC2 eluted later in the gradient than any remaining fusion protein. Analysis of the purified protein by SDS-polyacrylamide gel electrophoresis (see Fig. 2) showed the presence of a major polypeptide band corresponding to the expected molecular mass for YDC2 (30 kDa) and a faint band with a smaller molecular mass.

Determination of protein concentration. The concentration of pure YDC2 was estimated by a dye-binding assay (4), with bovine serum albumin as a standard. By comparison with the absorbance of the protein at 280 nm, an extinction coefficient ($\varepsilon^{0.1\%}_{280nm}$) of 1.5 was estimated. All YDC2 protein concentrations have been calculated for a dimer of the enzyme.

Oligonucleotide synthesis. Oligonucleotides were synthesized on a 394 DNA/RNA synthesizer (Applied Biosystems) and purified by gel electrophoresis in 12% polyacrylamide–7 M urea, the bands were excised, and DNA was eluted and recovered by ethanol precipitation. Oligonucleotides were radioactively labelled at their 5′ termini with [γ-^{32}P]ATP and T4 polynucleotide kinase (Amersham).

Assembly of four-way DNA junctions. Stoichiometric quantities of three unlabelled strands and one 5′-^{32}P-labelled strand were annealed by incubation in 50 mM Tris · HCl (pH 7.6)–10 mM $MgCl_2$–5 mM DTT–0.1 mM spermidine–0.1 mM EDTA for 3 min at 85°C followed by slow cooling. The assembled junctions were purified by gel electrophoresis in 5% polyacrylamide gels. The bands were excised, and DNA was recovered by electroelution.

(i) Junction 1. Junction 1 is a fixed junction with 20 bp in each arm with the central sequence of junction 1 (7); it was assembled from the following four oligonucleotides, each of 40 nucleotides: b strand (5′ AGGGATCCGTCCTAG CGTGCCGCTGCTACCGGAAGCTTCT 3′), h strand (5′ AGAAGCTTCCG GTAGCAGCGAGAGCGGTGGTTGAATTCCT 3′), r strand (5′ AGGAATT CAACCACCGCTCTTCTCGGCTGCAGTCTAGACT 3′), and x strand (5′ AG TCTAGACTGCAGCCGAGAGCACGCTAGGACGGATCCCT 3′). A duplex competitor for binding experiments was assembled by hybridization of the above b strand to its complement.

(ii) Junction 3. Junction 3 is a fixed junction with 25 bp in each arm, having the central sequence of junction 3 as described previously (7).

(iii) Junction Jbm4. Junction Jbm4 is a junction with 15 bp in each arm that can branch migrate over 12 steps. It was assembled from the following oligonucleotides: a strand (5′ GGAATCATC**CACTCGCCTTAA**CACTGCGTT 3′), b strand (5′ AACGCAGTG**TTAAGGCGAGTG**ATCCAGGTT 3′), c strand (5′ AACCTGGAT**CACTCGCCTTAA**TCGCTTGCG 3′), d strand (5′ CGCAAG CGA**TTAAGGCGAGTG**GATGATTCC 3′). Nucleotides highlighted in boldface type comprise the branch-migrating core of the junction.

Gel electrophoretic retardation analysis of YDC2-DNA junction interactions. Samples of purified MBP-YDC2 or YDC2 protein were incubated with 5′-^{32}P-labeled four-way DNA junctions in binding buffer (20 mM Tris [pH 8.0], 200 mM

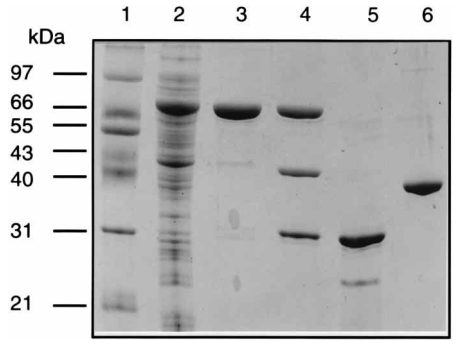

FIG. 2. Polyacrylamide gel electrophoretic analysis of YDC2 purification. Lanes: 1, protein molecular mass standards (molecular masses in kilodaltons are shown on left); 2, crude lysate of *E. coli* DH5αF′I^q overexpressing the MBP-YDC2 fusion protein; 3, MBP-YDC2 fusion protein after purification on amylose and HS columns; 4, MBP-YDC2 fusion partially cleaved with factor Xa to release the YDC2 protein; 5, cleaved YDC2 after purification on the HS column; 6, pure CCE1 protein.

NaCl, 0.2 mM DTT, 5 mM EDTA, 0.1 mg of bovine serum albumin per ml, calf thymus duplex competitor DNA where stated) in a 10-μl total volume for 5 min at 20°C, prior to addition of one-sixth volume of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll type 400). Samples were loaded onto 5% polyacrylamide gels and electrophoresed in Tris-borate-EDTA (TBE) buffer. After electrophoresis, the gels were dried on Whatman 3MM paper and exposed to X-ray film for documentation or storage phosphor screens for quantification on a 400S PhosphorImager (Molecular Dynamics). In experiments to measure dissociation constants, data were analyzed as the fraction of DNA bound (f_b) versus protein concentration and were fitted by nonlinear regression analysis to the equation

$$f_b = \frac{(1 + K_A P_T + K_A D_T) - [(1 + K_A P_T + K_A D_T)^2 - (4 D_T K^2{}_A P_T)]^{1/2}}{2 D_T K_A}$$

where P_T is the total protein concentration (calculated for a dimer) and D_T is the total DNA concentration (11). At higher concentrations of YDC2, where a discrete supershifted band was visible, it was counted as bound DNA. For the purposes of the calculation, YDC2 was considered to be a tight dimer in solution. The dissociation constant (K_D) is the reciprocal of the association constant (K_A).

Cleavage of four-way junctions by resolving enzymes. Four-way junction DNA labelled with 5′ ^{32}P on the required strand (80 nM) was incubated with pure YDC2 (500 nM) in binding buffer in a 10-μl total volume at 37°C for 5 min. Reactions were initiated by the addition of $MgCl_2$ to 15 mM, the mixtures were incubated for 15 min at 37°C, and the reactions were stopped by the addition of an equal volume of loading buffer (0.1% bromophenol blue plus 0.1% xylene cyanol FF in deionized formamide) and heating to 95°C for 2 min followed by transfer to ice. DNA was analyzed on 0.4-mm-thick polyacrylamide gels in TBE buffer containing 7 M urea that were run hot to the touch (55°C). Following electrophoresis, the gels were processed as described above.

Sequencing of DNA. Cloned sequences were verified by primer extension with dideoxynucleoside triphosphates (27). For sequence markers used to analyze cleavage positions, chemical degradation sequencing with standard reactions followed by piperidine cleavage was used (20).

RESULTS

Identification, cloning, and expression of the *YDC2* gene. The *S. cerevisiae* CCE1 protein sequence was used as a probe to search for possible sequence homologs in the Swissprot database. A significant match was found with YDC2_SCHPO (accession no. Q01483), a hypothetical *S. pombe* protein encoded on chromosome 1 (Fig. 1). Although the *S. pombe* protein is 25% smaller than CCE1, the proteins are 28% identical. This level of sequence identity is suggestive though not definitive evidence of a common ancestral origin for the two proteins. The YDC2 gene was cloned by PCR amplification from *S. pombe* genomic DNA and was inserted in the vector pUC119. DNA sequencing confirmed the sequence in the data bank. The YDC2 gene was subcloned into the expression plasmid pMALc2, allowing expression of the YDC2 protein as a fusion with MBP in *E. coli*. The overexpressed fusion protein was purified by chromatography on amylose and POROS HS columns. The YDC2 protein was liberated from the MBP fusion by digestion with the protease factor Xa and was purified on a POROS HS column. YDC2 produced in this manner has the 9-amino-acid N-terminal extension ISEFGSHMA compared to the theoretical sequence. The purification of the YDC2 protein is summarized in Fig. 2.

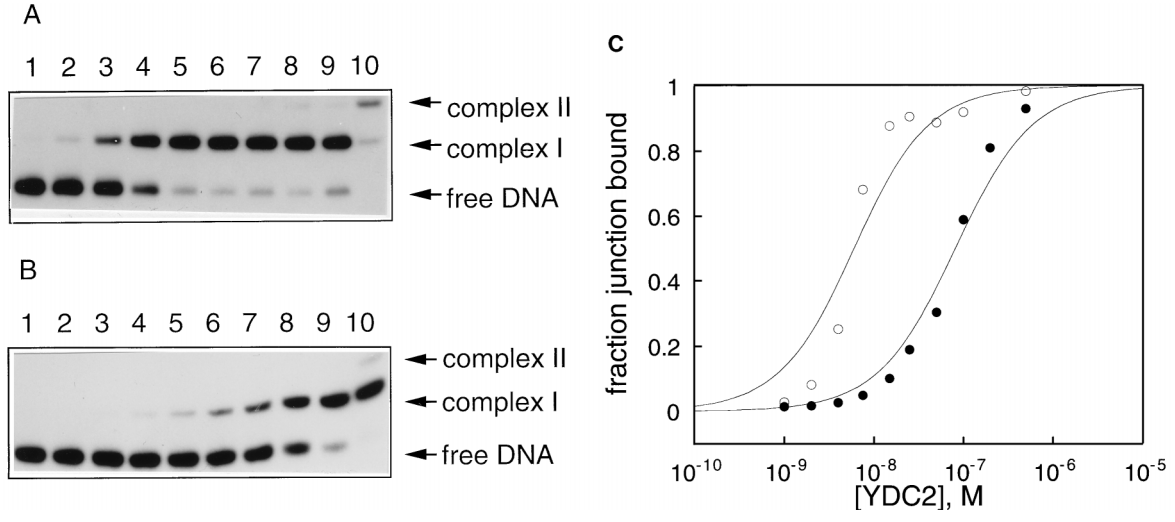

FIG. 3. Binding of YDC2 to a four-way DNA junction. (A and B) Electrophoretic analysis of binding. 5′-^{32}P-labelled junction 1 (1 nM) was incubated with increasing concentrations of YDC2 at room temperature in binding buffer in the absence (A) or presence (B) of a 1,400-fold (wt/wt) excess of calf thymus DNA competitor. Free junction and DNA-junction complexes were separated by electrophoresis in 5% acrylamide–TBE gels and visualized by autoradiography. YDC2 concentrations used in both panels A and B were as follows: lane 1, 1 nM; lane 2, 2 nM; lane 3, 4 nM; lane 4, 7.5 nM; lane 5, 15 nM; lane 6, 25 nM; lane 7, 50 nM; lane 8, 100 nM; lane 9, 200 nM; lane 10, 500 nM. (C) Binding isotherms for the interaction of YDC2 in the presence and absence of competitor DNA. The fraction of DNA junction bound to protein was calculated for each concentration of YDC2 by phosphorimaging and plotted against the logarithm of the protein molarity (with YDC2 assumed to be dimeric). The data were fitted to a model for the binding process (see Materials and Methods), from which the binding affinities were calculated. Symbols: ○, binding in the absence of competitor; ●, binding in the presence of competitor.

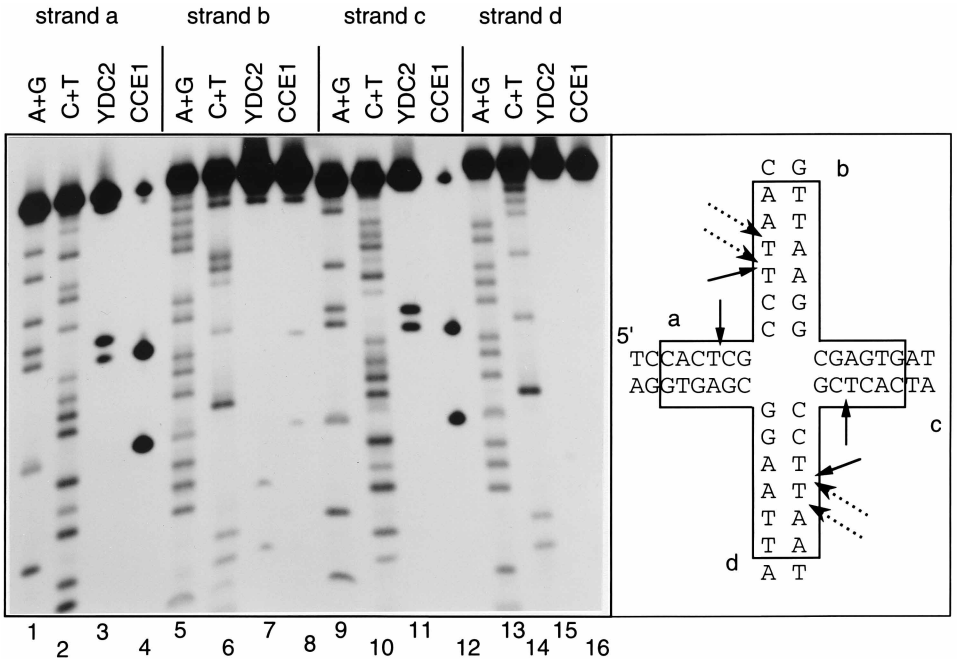

FIG. 4. Sequence dependence of cleavage of a four-way DNA junction by YDC2. The panel on the right shows the core of junction Jbm4, a junction with arms of 15 bp, which can undergo 12 steps of branch migration (boxed). The four strands of this junction are labelled a, b, c, and d as indicated. Four versions of this junction were prepared, with each of the strands individually 5′-^{32}P labelled. These were incubated with YDC2 or CCE1 in binding buffer supplemented with 15 mM $MgCl_2$ at 37°C for 15 min, and the products were separated by denaturing gel electrophoresis and detected by autoradiography. Cleavage was detected by both YDC2 and CCE1 on the a and c strands, at positions indicated by arrows on the junction sequence (solid arrows, CCE1; broken arrows, YDC2). Lanes: 1 and 2, sequence markers derived from chemical cleavage of the a strand by formate and hydrazine, respectively; 3 and 4, cleavage of the a strand by YDC2 and CCE1, respectively; 5 and 6, sequence markers derived from chemical cleavage of the b strand by formate and hydrazine, respectively; 7 and 8, cleavage of the b strand by YDC2 and CCE1, respectively; 9 and 10, sequence markers derived from chemical cleavage of the c strand by formate and hydrazine, respectively; 11 and 12, cleavage of the c strand by YDC2 and CCE1, respectively; 13 and 14, sequence markers derived from chemical cleavage of the d strand by formate and hydrazine, respectively; 15 and 16, cleavage of the d strand by YDC2 and CCE1, respectively.

YDC2 binds four-way DNA junctions. As a first test of our hypothesis that the YDC2_SCHPO gene product was a homolog of CCE1, the affinity of the purified protein for a four-way DNA junction was tested. Different amounts of protein were incubated with a 5′-^{32}P-labelled four-way junction in binding buffer with and without competitor DNA, and the bound and free junction species were separated by nondenaturing gel electrophoresis and detected by autoradiography. Incubation of junction with increasing concentrations of YDC2 gave rise to a well-defined retarded complex. At higher concentrations of protein, a second, more retarded complex was apparent (Fig. 3A). When the binding experiment was repeated in the presence of a 1,400-fold (wt/wt) excess of calf thymus competitor DNA, the first and second retarded complexes were still apparent (Fig. 3B). The fraction of DNA bound for each concentration of YDC2 was determined by phosphorimaging and plotted against the logarithm of the protein molarity (Fig. 3C). The data were fitted to a model for the binding process, from which estimates of the dissociation constants of YDC2 for the DNA junction were obtained. The dissociation constant (K_D) was calculated as 5 and 50 nM in the absence and presence of competitor, respectively. Thus, the YDC2 protein has a strong binding specificity for the structure of the four-way junction. By comparison, CCE1 has a K_D of approximately 1 nM under similar conditions in the absence of competitor DNA (43). The data for YDC2 binding without competitor give a sharper transition than was predicted by a simple two-state binding model. This may be due to the presence of a monomer-dimer equilibrium for YDC2 in solution (see below).

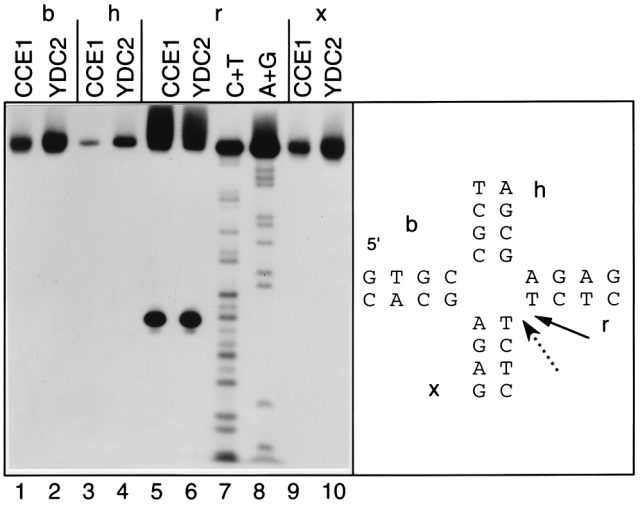

FIG. 5. Cleavage of a fixed four-way DNA junction by YDC2. The panel on the right shows the core of junction 1, a fixed junction with arms of 20 bp. Four versions of this junction were prepared, with each of the strands individually 5′-^{32}P labelled. These were incubated at 37°C for 15 min with YDC2 or CCE1 in binding buffer supplemented with 15 mM $MgCl_2$, and the products were separated by denaturing gel electrophoresis and detected by autoradiography. Both YDC2 and CCE1 cut at a single site on the r strand, which corresponds to cleavage at a 5′-CT site at the point of strand exchange, and are indicated by the arrows on the junction sequence (solid, CCE1; broken, YDC2). Lanes: 1 and 2, cleavage of the b strand by CCE1 and YDC2, respectively; 3 and 4, cleavage of the h strand by CCE1 and YDC2, respectively; 5 and 6, cleavage of the r strand by CCE1 and YDC2, respectively; lanes 7 and 8, sequence markers derived from chemical cleavage of the r strand by formate and hydrazine, respectively; 9 and 10, cleavage of the x strand by CCE1 and YDC2, respectively.

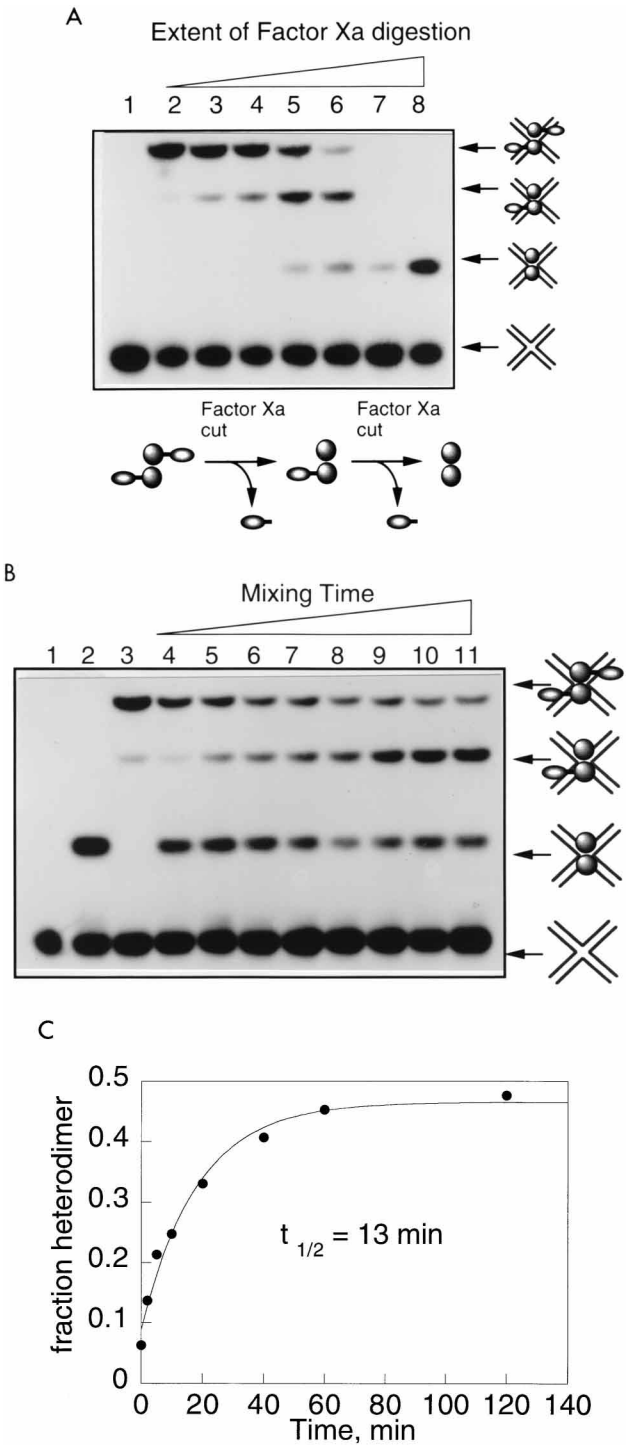

FIG. 6. Investigation of the quaternary structure of YDC2 by gel electrophoresis. (A) Analysis of the quaternary structure of YDC2 bound to a four-way junction. MBP-YDC2 fusion protein was cleaved by factor Xa protease to increasing extents, the resultant protein was incubated with a 5′-^{32}P-labelled junction in the presence of 500-fold (wt/wt) excess of competitor DNA, and the products were separated by nondenaturing gel electrophoresis and visualized by autoradiography. The extent of junction retardation is dependent on the mass of protein bound, with MBP-YDC2 giving a greater retardation than cleaved YDC2. The transient appearance of a complex with intermediate mobility is indicative of a dimeric structure for YDC2 bound to a four-way junction, as indicated in the schematic on the right. The representation of the four-way junction in the schematic is not intended to represent the actual structure of the junction. MBP-YDC2 (10^{-7} M) was incubated overnight with factor Xa at room temperature prior to incubation with the four-way junction. Lane 1 contains junction DNA without added protein; other lanes have MBP-YDC2 incubated with the following amounts of factor Xa: lane 2, 0 μg; lane 3, 0.1 μg; lane 4, 0.2 μg; lane 5, 0.5 μg; lane 6, 1 μg; lane 7, 5 μg; lane 8, 10 μg. (B) Subunit exchange in free solution. Pure MBP-YDC2 and YDC2 were mixed for various times in binding buffer, prior to the addition of 5′-^{32}P-labelled junction in the presence of a 500-fold (wt/wt) excess of competitor DNA. Complexes were separated by nondenaturing gel electrophoresis and visualized by autoradiography. The time-dependent increase in the proportion of the heterodimeric complex is indicative of subunit mixing in the timescale of the experiment. Lane 1, free DNA; lane 2, junction incubated with YDC2 alone; lane 3, junction incubated with MBP-YDC2 alone; other lanes, junction incubated with YDC2 and MBP-YDC2 that had been mixed for 0 min (lane 4), 2 min (lane 5), 5 min (lane 6), 10 min (lane 7), 20 min (lane 8), 40 min (lane 9), 60 min (lane 10), or 120 min (lane 11). (C) The data from panel B were quantified by phosphorimaging, and the ratio of radioactivity in the band corresponding to the heterodimer to that in all retarded species was plotted as a function of mixing time. Slow mixing of subunits was observed, with equilibrium achieved in approximately 60 min. The presence of heterodimer at time zero may be due to the presence of a small quantity of heterodimer in the MBP-YDC2 preparation (panel B, lane 3), suggesting that little or no subunit exchange occurs in the 5 min between addition of the DNA and gel loading. This would be the case if the rate of binding the junction (k_{on}) is much higher than the rate of subunit exchange. The data were fitted with an empirical simple exponential, giving a half-time for the exchange process of approximately 13 min under the stated experimental conditions.

YDC2 cuts a branch-migrating four-way DNA junction. We next wanted to determine whether the YDC2 protein possessed endonuclease activity. The cellular resolving enzymes RuvC and CCE1 display sequence-specific endonuclease activity; CCE1 shows a marked preference for cleavage after 5′-CT sequences (42), while RuvC has a 5′-TT cleavage consensus sequence (29). Both enzymes introduce paired nicks in four-way junctions at the point of strand exchange (42). We therefore designed a synthetic four-way DNA junction that was capable of 12 steps of branch migration (Jbm4). The sequence of the mobile core of Jbm4 was designed so that every possible dinucleotide sequence combination could be present at the point of strand exchange (Fig. 4), with the aim of ensuring that sequences favorable for cleavage should exist for any resolving enzymes with a degree of sequence specificity similar to CCE1 and RuvC. Junction Jbm4 individually labelled on each of its four strands was incubated with CCE1 or YDC2 under standard cleavage conditions, and the products were separated by denaturing gel electrophoresis followed by autoradiography (Fig. 4). As expected, CCE1 introduced paired cuts in the a and c strands at two 5′-CT sequences. YDC2 cleaved the junction at one of the two 5′-CT sequences recognized by CCE1 and at an adjacent 5′-TT sequence (Fig. 4). In addition, both enzymes introduced different weak, unpaired cuts in the b strand of the junction. YDC2 is therefore a junction-resolving enzyme and appears to possess a sequence specificity that is overlapping but distinct from both CCE1 and RuvC.

YDC2 cuts a fixed four-way DNA junction at the point of strand exchange. Incubation of four-way junctions capable of branch migration with resolving enzymes allows a range of nucleotide sequences to be scanned and favorable cleavage sequences to be defined but provides no information on the position of strand cleavage relative to the junction center. CCE1 cleaves fixed junctions at or 1 nucleotide 3′ of the point of strand exchange (42). In each case, cleavage occurs at the recognition sequence 5′-CT. In common with CCE1, YDC2 introduced a single cut at a 5′-CT site in the r strand of junction 1, at the point of strand exchange (Fig. 5). However, another fixed junction cut by CCE1, junction 3, is not cleaved by YDC2 (data not shown). The data suggest that YDC2 prefers to cut immediately at the point of strand exchange of four-way junctions, in common with CCE1 and RuvC (3).

Quaternary structure of YDC2. The quaternary structure of YDC2, both bound to the junction and in free solution, was investigated by methods first developed for the analysis of CCE1 (42). These experiments take advantage of the finding that the MBP-YDC2 fusion and the cleaved YDC2 protein retard four-way junctions to different extents in native gel electrophoresis. In the first experiment, MBP-YDC2 fusion was subjected to limited cleavage by factor Xa protease and the resultant protein mixture was used in an electrophoretic retardation experiment with a radioactive four-way junction (Fig. 6A). As the extent of digestion proceeded, the complex corresponding to the bound fusion protein decreased and that due to the cleaved YDC2 protein increased, as expected. However, a third complex, with intermediate mobility, was observed to appear and subsequently disappear during the digestion. The simplest explanation for this observation is that the YDC2 protein binds to a four-way junction as a dimer; thus, the intermediate species would correspond to the situation where one subunit of the dimer has had the MBP fusion removed by factor Xa while the other remained intact.

The quaternary structure of YDC2 in free solution was examined in a further experiment in which samples of purified fusion and native proteins were mixed for a set time prior to addition of the four-way junction and separation of the bound and free species by gel electrophoresis. If YDC2 was a nondissociable dimer in solution, the band corresponding to the heterodimer would not be seen in this experiment. On the other hand, if YDC2 was monomeric in solution but dimerized on binding the four-way junction, a fixed proportion of the intermediate species would be expected, invariant with time. In fact, what is observed lies between these two extremes—a time-dependent increase in the proportion of heterodimer (Fig. 6B). This suggests that YDC2 is dimeric in solution and can undergo subunit exchange. The relative proportion of heterodimer that appears can be plotted against the mixing time, and the data can be fitted by a simple exponential function (Fig. 6C). The half-time for subunit mixing is estimated as 13 min under these conditions.

DISCUSSION

YDC2 has strong specificity for four-way DNA junctions. We have demonstrated that the YDC2 protein of *S. pombe* is a Holliday junction endonuclease. Since this is only the second such enzyme from a eukaryotic organism to be characterized, it provides valuable insights into the generality of the properties of the four-way junction-resolving enzymes. In common with all other junction-resolving enzymes studied so far, YDC2 binds Holliday junctions with a very strong preference for the four-way junction structure over duplex competitor DNA. A high degree of structural specificity would be vital in vivo: in the cellular milieu, the Holliday junction is likely to be a very rare structure in the midst of a vast amount of nucleoprotein. YDC2 binds junctions as a dimer and exists in a monomer-dimer equilibrium in solution, as has been found for CCE1 (42), T4 endonuclease VII (25), and RuvC (30). In contrast, T7 endonuclease I is a nondissociable dimer in solution (24). In studies with a cruciform substrate stabilized by negative supercoiling, the subunits of both T4 endonuclease VII and T7 endonuclease I have been shown to act nearly simultaneously (both cleaving within the lifetime of the protein-junction complex) but independently in junction cleavage (12, 24).

YDC2 is a sequence-specific endonuclease. The two cellular resolving enzymes studied to date, RuvC and CCE1, both exhibit a sequence specificity for junction cleavage which overlays the structure-specific binding properties. The consensus sequence for RuvC cleavage has been defined as 5′-(A/T)TT/(G/C) (28), with specificity residing predominantly at the thymidine base immediately 5′ of the cleavage site (32). CCE1 cuts a range of mobile and fixed four-way DNA junctions with a clear preference for 5′-CT sequences, and single-turnover kinetic analysis has demonstrated a 30-fold decrease in the cleavage rate when the target cytosine base is replaced with a guanine (42). Bases both 5′ and 3′ to the 5′-CT consensus sequence also influence the rate of junction cleavage (27a). Since a dinucleotide appears to provide a sufficient target sequence for CCE1 and RuvC, we designed a mobile four-way junction, Jbm4, which contains every possible combination of dinucleotide sequences in the mobile core and thus allows resolving enzymes to locate preferred cleavage sites. YDC2 cleaves Jbm4 at two sites in the a and c arms with the sequences 5′-CT and 5′-TT. However, additional 5′-CT and 5′-TT sequences present in the core are not cleaved; therefore, the sequence specificity of YDC2 is probably more complex than a simple dinucleotide sequence. Analysis of the cleavage of a fixed junction by YDC2 reveals that the enzyme cuts one strand at the junction center at a 5′-CT site. The opposing strand remains uncleaved since no target DNA sequence is present. Thus, as with CCE1 (42) and RuvC (30, 32), cleavage of fixed junctions by YDC2 requires the correct positioning of favorable sequences with respect to the point of strand exchange; neither the ability to branch migrate nor the presence of homologous DNA sequences on opposing arms is essential for activity. A detailed understanding of the mechanism by which these endonucleases recognize both the structure of the four-way junction and specific nucleotide cleavage sequences remains a strong focus for future study.

Significance for mitochondrial recombination. Given that YDC2 and CCE1 are clear structural homologs and display many similar biochemical characteristics, it seems likely that the YDC2 enzyme is the functional equivalent of CCE1, i.e., that it plays a role in mtDNA recombination in *S. pombe*. Homologous recombination is well documented in the mitochondria of fungi, higher plants, and protists (13) but has been considered to be rare or absent in animal mitochondria (23, 38). However, recent reports suggest that human mitochondria can fuse and interact (36) and that homologous recombination can occur in the mitochondria of metazoa (18, 37). The elucidation of the mechanism of homologous mtDNA recombination is of more than academic interest; many human diseases are associated with deletions and rearrangements of mtDNA (39), and the accumulation of mtDNA mutations correlates with aging in humans, perhaps playing a causal role in senescence (21, 39). Although both are ascomycetes, *S. pombe* and *S. cerevisiae* diverged very early in the evolution of the fungi, up to 1 billion years ago by some estimates, and many *S. pombe* protein sequences are as divergent from *S. cerevisiae* sequences as they are from those of higher eukaryotes (33). The preservation of the CCE1/YDC2 Holliday junction endonuclease in these two highly divergent organisms may indicate a conserved pathway for the homologous recombination of mtDNA in fungi, higher plants, protists, and perhaps metazoa.

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REFERENCES

1. **Ariyoshi, M., D. G. Vassylyev, H. Iwasaki, H. Nakamura, H. Shinagawa, and K. Morikawa.** 1994. Atomic structure of the RuvC resolvase: a Holliday junction-specific endonuclease from *E. coli*. Cell **78:**1063–1072.

2. **Bennett, R. J., and S. C. West.** 1995. Structural analysis of the RuvC-Holliday junction complex reveals an unfolded junction. J. Mol. Biol. **252:**213–226.
3. **Bennett, R. J., and S. C. West.** 1996. Resolution of Holliday junctions in genetic recombination: RuvC protein nicks DNA at the point of strand exchange. Proc. Natl. Acad. Sci. USA **93:**12217–12222.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. Anal. Biochem. **72:**248–254.
5. **de Massey, B., R. A. Weisberg, and F. W. Studier.** 1987. Gene 3 endonuclease of bacteriophage T7 resolves conformationally branched structures in double-stranded DNA. J. Mol. Biol. **193:**359–376.
6. **Duckett, D. R., M. J.-E. Giraud-Panis, and D. M. J. Lilley.** 1995. Binding of the junction-resolving enzyme bacteriophage T7 endonuclease I to DNA: separation of binding and catalysis by mutation. J. Mol. Biol. **246:**95–107.
7. **Duckett, D. R., A. I. H. Murchie, S. Diekmann, E. von Kitzing, B. Kemper, and D. M. J. Lilley.** 1988. The structure of the Holliday junction and its resolution. Cell **55:**79–89.
8. **Elborough, K. M., and S. C. West.** 1990. Resolution of synthetic Holliday junctions in DNA by an endonuclease from calf thymus. EMBO J. **9:**2931–2936.
9. **Evans, D. H., and R. Kolodner.** 1988. Effect of DNA structure and nucleotide sequence on Holliday junction resolution by a *Saccharomyces cerevisiae* endonuclease. J. Mol. Biol. **201:**69–80.
10. **Ezekiel, U. R., and H. P. Zassenhaus.** 1993. Localization of a cruciform cutting endonuclease to yeast mitochondria. Mol. Gen. Genet. **240:**414–418.
11. **Giraud-Panis, M.-J. E., and D. M. J. Lilley.** 1996. T4 endonuclease VII: importance of a histidine-aspartate cluster within the zinc-binding domain. J. Biol. Chem. **271:**33148–33155.
12. **Giraud-Panis, M.-J. E., and D. M. J. Lilley.** 1997. Near-simultaneous DNA cleavage by the subunits of the junction resolving enzyme T4 endonuclease VII. EMBO J. **16:**2528–2534.
13. **Gray, M. W.** 1989. Origin and evolution of mitochodrial DNA. Annu. Rev. Cell Biol. **5:**25–50.
14. **Hyde, H., A. A. Davies, F. E. Benson, and S. C. West.** 1994. Resolution of recombination intermediates by a mammalian activity functionally analogous to *Escherichia coli* RuvC resolvase. J. Biol. Chem. **269:**5202–5209.
15. **Kleff, S., B. Kemper, and R. Sternglanz.** 1992. Identification and characterisation of yeast mutants and the gene for cruciform cutting endonuclease. EMBO J. **11:**699–704.
16. **Kupfer, C., and B. Kemper.** 1996. Reactions of mitochondrial cruciform cutting endonuclease I (CCE1) of yeast *Saccharomyces cerevisiae* with branched DNAs *in vitro*. Eur. J. Biochem. **238:**77–87.
17. **Lockshon, D., S. G. Zweifel, L. L. Freeman-Cook, H. E. Lorimer, B. J. Brewer, and W. L. Fangman.** 1995. A role for recombination junctions in the segregation of mitochondrial DNA in yeast. Cell **81:**947–955.
18. **Lunt, D. H., and B. C. Hyman.** 1997. Animal mitochondrial DNA replication. Nature **387:**247.
19. **Mahdi, A. A., G. J. Sharples, T. N. Mandal, and R. G. Lloyd.** 1996. Holliday junction resolvases encoded by homologous *rusA* genes in *Escherichia coli* K-12 and phage 82. J. Mol. Biol. **257:**561–573.
20. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
21. **Miquel, J.** 1992. An update on the mitochondrial-DNA hypothesis of cell aging. Mutat. Res. **275:**209–216.
22. **Mizuuchi, K., B. Kemper, J. Hays, and R. A. Weisberg.** 1982. T4 endonuclease VII cleaves Holliday structures. Cell **29:**357–365.
23. **Moritz, C., T. E. Dowling, and W. M. Brown.** 1987. Evolution of animal mitochondrial-DNA—relevance for population biology and systematics. Annu. Rev. Ecol. Syst. **18:**269–292.
24. **Parkinson, M. J., and D. M. J. Lilley.** 1997. The junction-resolving enzyme T7 endonuclease I: quaternary structure and interaction with DNA. J. Mol. Biol. **270:**169–178.
25. **Pöhler, J. R. G., M.-J. E. Giraud-Panis, and D. M. J. Lilley.** 1996. T4 endonuclease VII selects and alters the structure of the four-way DNA junction: binding of a resolution-defective mutant enzyme. J. Mol. Biol. **260:**678–696.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

27a. **Schofield, M., D. M. J. Lilley, and M. F. White.** Unpublished data.

28. **Shah, R., R. J. Bennett, and S. C. West.** 1994. Activation of RuvC Holliday junction resolvase in vitro. Nucleic Acids Res. **22:**2490–2497.
29. **Shah, R., R. J. Bennett, and S. C. West.** 1994. Genetic recombination in *E. coli*: RuvC protein cleaves Holliday junctions at resolution hotspots in vitro. Cell **79:**853–864.
30. **Shah, R., R. Cosstick, and S. C. West.** 1997. The RuvC protein dimer resolves Holliday junctions by a dual incision mechanism that involves base-specific contacts. EMBO J. **16:**1464–1472.
31. **Sharples, G. J., and R. G. Lloyd.** 1991. Resolution of Holliday junctions in *Escherichia coli*: identification of the *ruvC* gene product as a 19-kilodalton protein. J. Bacteriol. **173:**7711–7715.
32. **Shida, T., H. Iwasaki, A. Saito, Y. Kyogoku, and H. Shinagawa.** 1996. Analysis of substrate specificity of the RuvC Holliday junction resolvase with synthetic Holliday junctions. J. Biol. Chem. **271:**26105–26109.
33. **Sipiczki, M.** 1989. Taxonomy and phylogenesis, p. 437–448. *In* A. Nasim, P. Young, and B. F. Johnson (ed.), Molecular biology of the fission yeast. Academic Press, Inc., San Diego, Calif.
34. **Stuart, D., K. Ellison, K. Graham, and G. McFadden.** 1992. In vitro resolution of poxvirus replicative intermediates into linear minichromosomes with hairpin termini by a virally induced Holliday junction endonuclease. J. Virol. **66:**1551–1563.
35. **Symington, L., and R. Kolodner.** 1985. Partial purification of an endonuclease from *Saccharomyces cerevisiae* that cleaves Holliday junctions. Proc. Natl. Acad. Sci. USA **82:**7247–7251.
36. **Takai, D., K. Inoue, Y.-I. Goto, I. Nonaka, and J.-I. Hayashi.** 1997. The interorganellar interaction between distinct human mitochondria with deletion mutant mtDNA from a patient with mitochondrial disease and with HeLa mtDNA. J. Biol. Chem. **272:**6028–6033.
37. **Thyagarajan, B., R. A. Padua, and C. Campbell.** 1996. Mammalian mitochondria possess homologous DNA recombination activity. J. Biol. Chem. **271:**27536–27543.
38. **Wallace, D. C.** 1992. Diseases of the mitochondrial DNA. Annu. Rev. Biochem. **61:**1175–1212.
39. **Wallace, D. C., J. M. Shoffner, I. Trounce, M. D. Brown, S. W. Ballinger, M. Corraldebrinski, T. Horton, A. S. Jun, and M. T. Lott.** 1995. Mitochondrial-DNA mutations in human degenerative diseases and aging. Biochim. Biophys. Acta **1271:**141–151.
40. **West, S. C., and A. Korner.** 1985. Cleavage of cruciform DNA structures by an activity from *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **82:**6445–6449.
41. **White, M. F., M.-J. E. Giraud-Panis, J. R. G. Pöhler, and D. M. J. Lilley.** 1997. Recognition and manipulation of branched DNA structures by junction resolving enzymes. J. Mol. Biol. **269:**647–664.
42. **White, M. F., and D. M. J. Lilley.** 1996. The structure-selectivity and sequence-preference of the junction-resolving enzyme CCE1 of *Saccharomyces cerevisiae*. J. Mol. Biol. **257:**330–341.
43. **White, M. F., and D. M. J. Lilley.** 1997. The resolving enzyme CCE1 of yeast opens the structure of the four-way DNA junction. J. Mol. Biol. **266:**122–134.
44. **Zweifel, S. G., and W. L. Fangman.** 1991. A nuclear mutation reversing a biased transmission of yeast mitochondrial DNA. Genetics **128:**214–249.